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Full Length Research Paper

Characterization of *Metarhizium anisopliae* using amplifed ribossomal DNA restriction analysis (ARDRA) and internal transcribed spacer (ITS) sequence analysis

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Molecular methods have revolutionized systematic entomology in the genus *Metarhizium*. In Brazil, the importance of these entomopathogenic fungi in agriculture is critical to the control of the sugar cane spittlebug (*Mahanarva posticata*) (Homoptera: Cercopidae). Other important factors for the selection of isolates of *Metarhizium anisopliae* for use in the biological control of particular pests are virulence, reproductive characteristics and the media of artificial cultures. In addition, the genetic variability of these entomopathogenic fungi indicates a great potential for pest control and a lack of a link between isolates from specific host sites and fungal virulence. We studied five *Metarhizium* hosts including the sugar cane spittlebug (IPA213, IPA215, IPA219 and IPA216) and the grasshopper (IPA217). It is noteworthy that these five isolates are used as biological control for growing sugar cane on an industrial scale for the Brazilian Northeast. Hence, the importance to genetically characterize these isolates of *Metarhizium* cannot be over emphasized. Amplifed ribossomal DNA restriction analysis (ARDRA) did not differentiate any of the isolates that were studied following digestion with three endonuclease namely; *Hind*III, *Hinf*I and *Hpa*I. The data that were generated using ITS1 and ITS4 markers revealed that although these isolates infect the same host, they are genetically distinct perhaps due to the unknown origin of the hosts. However, the isolate IPA217, which was isolated from the grasshopper, did not behave differently from the others. These results show that the *M. anisopliae* complex is monophyletic and it was concluded that the use of markers and morphological studies yielded results that may corroborate the idea that investigation of the phenotypic and molecular characteristics of this genus may indentify a new species of *Metarhizium*.

Key words: *Cordyceps brittebankisoides*, genetic identity, ITS1, ITS4, phylogeny, sugar cane spittlebug.

INTRODUCTION

The entomopathogenic fungus *Metarhizium anisopliae* (Metschnikoff) Sorokin has been isolated from many insect species and is a biocontrol agent that infects a wide variety of pests including the orders Lepidoptera, Coleoptera, Orthoptera and Hemiptera (Pria Junior et al., 2008). *M. anisopliae* is used commercially due to this property of infection in a broad spectrum of hosts. In Brazil, *Mahanarva posticata* (Homoptera: Cercopidae) has been used to control the sugar cane spittlebug. It is used to control a variety of pests in other countries such as Australia, U.S.A and Colombia (Miller et al., 2004). A

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Abbreviations: ITS, Internal transcribed spacer; **PCR,** polymerase chain reaction.

previous study evaluated the efficiency of *M. anisopliae* isolates in the control of *Mahanarva fimbriolata* on sugarcane in Northeastern Brazil. The isolates that control *M. posticata* showed a large genetic variability and adaptability (Batista et al., 2002). These two properties are not favorable in terms of maximized efficiency in these micro-organisms in the original isolation sites (Almeida et al., 1997). Other important factors for the selection of isolates of *M*. *anisopliae* for biological control of particular pests are virulence, reproductive aspects and the media of artificial cultures. In addition, the genetic variability of these entomopathogenic fungi indicates a great potential for pest control and the lack of a link between isolate-host-site and fungal virulence (Almeida and Batista, 2001).

The genera of entomopathogenic fungi that are characterized as Deuteromycotina: Hyphomycetes includes several asexual fungi. Deuteromycota are fungi that do not fit into the commonly established taxonomic classifications of fungi that are based on biological species concepts or morphological characteristics of sexual structures because their sexual form of reproduction has never been observed. Therefore, Deuteromycota are known as "imperfect fungi." Only their asexual form of reproduction is known, meaning that these fungi produce their spores asexually. Currently, there are three genera *Metarhizium* and nine varieties. This first genus is *M. anisopliae*, which contains the following varieties: *M. anisopliae* (Metsch.) Sorokin, var*. anisopliae*, var. *majus*, var. *lepidiotum* (*lepidiotae*) and var. *acridum*. The second genus is *M. flavoviridae* Gams and Rozsypal, which contains the following varieties: var. *flaviride*, var. *minus,* var. *novazealandicum* and var. *pemphigum.* The third genus is called *Metarhizium album* Petch (Driver et al., 2002). Some pathogens of insects have no restrictions on their hosts and infect only a few species of insects, whereas others infect a wider range of hosts and include any of *M. anisopliae.* The taxonomy of these fungi has undergone great changes. Beginning in 2001, *M. anisopliae* var. *majus* was identified as the anamorphic form of the fungus *Cordyceps brittebankisoides* using the internal transcribed spacer (ITS) sequence. These data indicate *M. anisopliae* var. *majus* and *Cordyceps brittebankisoides* as one Ascomycota. A previous study investigated the phenotypic and molecular characteristics of a new Cordyceps species called *Cordyceps chongqingensis* sp. nov. The data have revealed a new species of *Metarhizium* using morphological observations under a microscope to visualize its anamorph form (Yang et al., 2009).

The analysis of the rDNA spacer region and comparative studies of nucleotide sequences of rDNA genes using the amplifed ribossomal DNA restriction analysis (ARDRA) technique (restriction analysis of ribosomal DNA) have been used to study the phylogeny and taxonomy of plants, fungi and bacteria (Schlotterer et al., 1994; Tian et al., 2009; Yadav et al., 2011). Although, the regions of ribosomal genes are highly conserved evolved. Therefore, these ITS regions may vary in intraspecific base sequence and length (Gerbi, 1985) and are often used for taxonomy of species and genera (Antoniolli et al., 2000; Gomes et al., 2002; Menezes et al., 2010). Previous studies have also observed similar findings for the sequences of 26S rDNA D1/D2 and ITS regions of the variants of *Metarhizium taii*, which were named *Metarhizium taii* var. c*hongqingensis* nov. A previous study using 33 isolates from these genera, including a broader sample of *Metarhizium* taxa, determined that ‘‘*Metarhizium anisopliae* var. *frigidum*’’ was more closely related to the *Metarhizium flavoviride* clade than the *M. anisopliae* clade (Bischoff et al., 2006).

The D3 region of the large ribosomal subunit (LSU) and ITS rDNA regions that were used in the aforementioned phylogenetic study do not support a clear distinction between ‘‘*Metarhizium anisopliae* var. *frigidum*’’ and *Metarhizium flavoviride*. Therefore, they are recognized as the same clade. Based on the rDNA ITS1-5.8S-ITS2 phylogeny, a previous study determined that the *M. anisopliae* complex is monophyletic (Driver et al., 2000). Similar results have been found in another study (Bischoff et al., 2009). The phylogenetic evidence and in some cases, morphological evidence, supports the monophyly of nine terminal taxa in the *M. anisopliae* complex that are recognized as species. We proposed to rank the species *M. anisopliae*, *M. guizhouense*, *M. pingshaense*, *M. acridum* stat. nov., *M. lepidiotae* stat. nov., and *M. majus* stat. nov. It was detected that *M. brunneum* described the new species *M. globosum* and *M. robertsii* and showed that *M. taii* is *M. guizhouense*. The application of systematic and phylogenetic research has practical effects in applied entomology. The objective of the present study was to assess the effects and elucidate the phylogeny of the isolates from the Laboratory of Biological Control of the Agronomical Institute of Pernambuco (IPA) in Brazil.

MATERIALS AND METHODS

A total of five isolates were used from the Laboratory of Biological Control of IPA in Brazil. Four isolates were obtained from the same host order Homoptera: Cercopedae (sugar cane spittlebug), whereas one isolate was obtained from the host IPA217 grasshopper (Orthoptera: Acrididae). For the long-term storage of the collected *Metarrhizia*, the colonies were transferred to Castellani media (Castellani, 1967) and stored at 4°C. For the DNA extraction, the mycelium was ground and added to 25 ml of liquid Becton and Dickinson (BD) medium for 72 h at 28°C without agitation. The samples were macerated with liquid nitrogen and subjected to the DNA easy Blood and Tissue Kit (QIAGEN) using the manufacturer’s instructions.

Electrophoresis was performed at 100 V using an agarose gel 0.8% in 0.5 × Tris borate EDTA (TBE) buffer containing bromothymol blue and staining with SybrGold (Invitrogen) were performed followed by quantification. Electrophoresis was run at 100 V, and the product was imaged using UV light-LPIX image EX

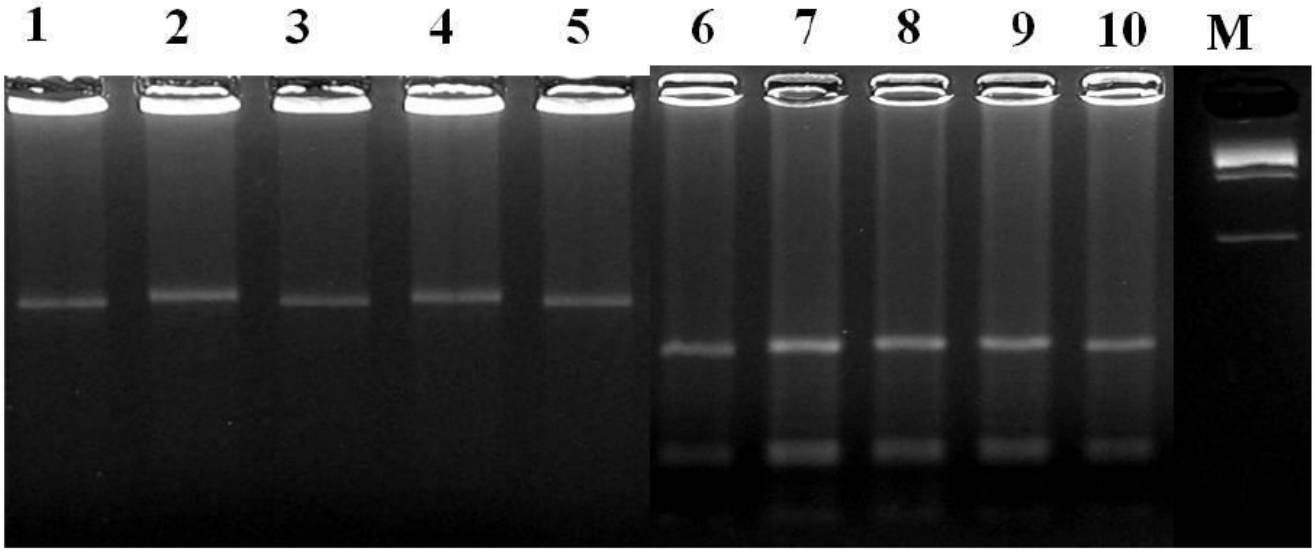

Figure 1. Restriction patterns of PCR-amplified fragments of ITS1-2 digested with restriction endonuclease *Hind*III (1 to 5) and *Hinf*I (6 to 10). 1 and 6, IPA213; 2 and 7, IPA215; 3 and 8, IPA219; 4 and 9, IPA216; 5 and 10, IPA217. M, *Eco*RI/*Hind*III bp ladder.

system (Loccus-Brazil). After amplification of the ITS region, the ITS1- and ITS4-amplified products were purified and cut with three restriction enzymes: *Hind*III, *Hinf*I and *Hpa*I. The purification consisted of adding 8 ml of 7.5 M ammonium acetate and 208 ml of 100% ethanol to a 1.5 ml-microtube, which was centrifuged at 9.000 g for 20 min at room temperature. The supernatant was discarded, and 150 ml of cold 70% ethanol was added followed by centrifugation for 5 min at 13,000 rpm. The ethanol was discarded, and the microtube was turned upside down onto a paper towel to dry overnight. Enzymatic digestion was performed using a final volume of 30 µl as follows: 8 µl of the amplified product, 10% of the buffer that was specific for each enzyme, 1 µl of each enzyme and 18 µl of ultrapure water. The samples were incubated for 12 h at the appropriate temperature for each enzyme. To visualize the cut products, we performed electrophoresis at 100 V using a 2% agarose gel that was stained with SybrGold (Invitrogen) and photographed as described earlier.

The ITS region was amplified using the primers ITS1 (5´-TCC GTA GGT GAA CCT GCG G-3´) and ITS4 (5´-TCC TCC GCT TAT TGA TAT GC-3´) (White et al., 1990). The sequencing was performed on the CENARGEN-EMBRAPA platform for DNA sequencing. The sequences were aligned in the program BioEdit v. 7.0.0. Phylogenetic analysis using the program Mega version 4.1 was performed. The database of the following gene sequences (GenBank) was used to assist in the classification based on the phylogenetic sequences of the ITS nucleotides: EF484924 and AY646386 (*M. anisopliae* var. *lepidiota* or *lepidiotum*); FJ787313 (*M. anisopliae* var. *acridum*); EU307926 and EU307906 (*M. anisopliae* var. *anisopliae*); AY375449 (*M. flavoviride*); AJ309333 (*Cordyceps brittlebankisoides*); AY375446 (*M. album)* and AY847486 (*M anisopliae* var. *majus*). Following the digestion with the restriction enzymes of *Hind*III, *Hinf*I and *Hpa*I, the amplified products of the region ITS1-2 exhibited no differences between the five isolates of *Metarhizium* collection of IPA. This result shows that the digestion of this ribosomal region does not generate products that can be used to differentiate between these entomopathogenic fungi (Figure 1). The results (Figure 2) showed that the origin of the host was not of great importance for the phylogeny and that the fungus IPA217 shared the same monophyletic branch to the other isolates that were from the cane sugar spittlebug. The genetic similarities of the fungi IPA213, IPA215 and IPA217 were more than 92% with *Metarhizium anisopliae* var. l*epidiotum*, and *lepidiota*, which was between them and had a genetic identity of 100%. These results show that different nomenclature is not necessary. The same result occurs with *M. anisopliae* var. *anisopliae*, which shares a monophyletic branch and 100% identity with *M. anisopliae* var. *acridum*. However, this similarity indicates their earliest ancestors. The fungus IPA217 showed an identity of 94% with *M. flavoviride* as well as a similarity above 92% and an identity of 94% with *Cordyceps brittlebankisoides* and *M. album*, respectively. The isolate IPA216 was most distinct from the other isolates. This finding indicates that IPA216 may be derived from an ancestor that was much older and shares an identity of 61% with *Metarhizium*. In addition, the isolate IPA217 may be an anamorph of *Cordyceps brittlebankisoides* or *Metarhizium album*. The results of the molecular analysis of the ITS region indicate that the isolates are *Metarhizium* but with a different ancestry and that these fungi may be new varieties that have not been previously identified. Further genetic, morphological and molecular studies are required to elucidate other characteristics of these isolates.

RESULTS AND DISCUSSION

The phylogeny of this genus is changing. In this study, the results show that molecular systematic studies may create significant changes in the taxonomy of these fungi. The elongation factor 1alpha (EF-1α), RNA polymerase II gene sequences (*RPB1*), RNA polymerase II gene (*RPB2*) and β-tubulin gene regions may be useful as phylogenetic markers. Interesting findings were observed by Bischoff et al. (2009) who posited that the 5' region of EF-1 α is very informative for use in routine identification of species within this genus. The results of several previous studies (Driver et al., 2000; Spatafora et al., 2007; Sung et al., 2007) as well as those of our current study have confirmed that *Metarhizium* is a monophyletic

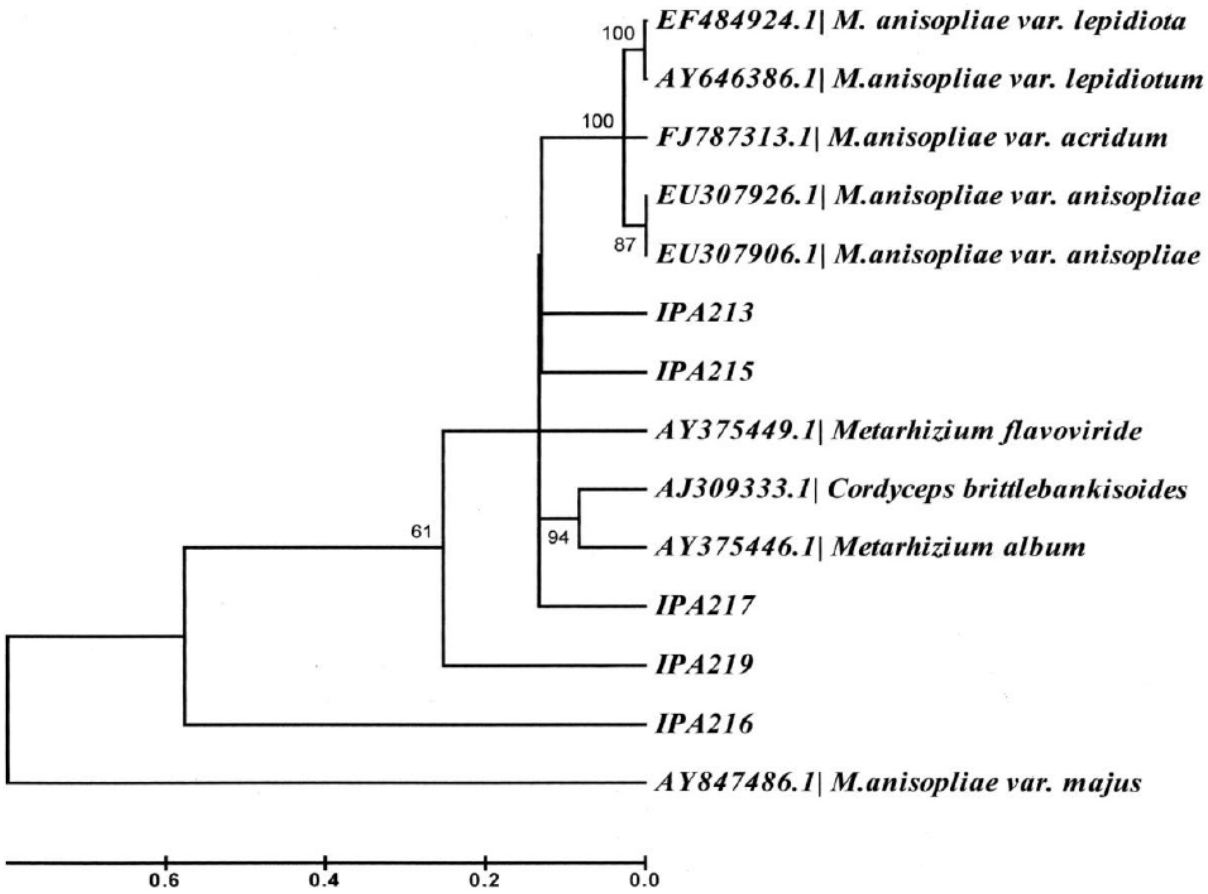

Figure 2. Phylogenetic tree based on ITS sequences from **Metarhizium** isolates using neighbor-joining (NJ) with Tamura-Nei and 1000 bootstrap tests.

group. All phylogenetic analyses of the *M. anisopliae* complex in this study used the closely related species *M. flavoviride*, *M. frigidum* and the out-group taxa. The markers ITS1 and ITS4 that were used in this study showed that the isolates IPA213, IPA215, IPA219 and IPA216, which were derived from the same host (sugar cane spittlebug), had different similarities and that the isolate IPA217, which was isolated from a different host (grasshopper), clustered with the majority of *Metarhizium*. Previous studies have noted the utility of the ribosomal ITS1 and ITS2 regions as targets for the molecular identification of fungi (Yang et al., 2009). The polymorphism of the ITS1 and ITS2 regions flanking the DNA sequence encoding the 5.8S rRNA is frequently used to identify *Cordyceps*. Although, we used the same published primers, which flank in different places, the genus *Cordyceps* showed a high genetic identity with the isolates IPA213 and IPA215. These results show that despite our inability to delimit the species based on morphological characteristics, the molecular data support the species recognition of these fungi. The two nuclear ribosomal genes have been extensively used in molecular systematics. These genes probably represent the most promising candidates for resolving relationships within the group.

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